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BFU-E Colony Growth in Response to Hydroxyurea: Correlation Between In Vitro and In Vivo Fetal Hemoglobin Induction

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Patients with sickle-cell anemia treated with hydroxyurea may have significant reduction in frequency and severity of pain episodes. However, previous clinical trials show a variable response to hydroxyurea. Criteria which can be used to select patients who are likely to respond to hydroxyurea treatment would be useful. Our laboratory has previously demonstrated an inverse linear relationship between the total number of burst-forming unit-erythroid (BFU-E) colonies and fetal hemoglobin levels in sickle-cell patients treated with hydroxyurea. In the present report, an in vitro cell culture system was established to evaluate the effects of hydroxyurea on BFU-E colony growth and induction of fetal hemoglobin production. Five Hb SS patients who were not previously treated with hydroxyurea and three Hb SS patients who failed to respond to hydroxyurea treatment were included in the study. The results show that the number of BFU-E colonies is decreased from 153.7 to 7.2 per 3×10^5 mononuclear cells, whereas fetal hemoglobin levels were increased from 5.1 to 19.4% in the presence of hydroxyurea in vitro in cultured erythroid progenitors, which were derived from 5 patients before treatment. The number of BFU-E colonies decreased from 153.7 to 2.0 per 3×10^5 mononuclear cells in the in vitro cultures obtained from serial peripheral blood samples over a 9- to 20-week period of oral hydroxyurea therapy. A simultaneous rise in fetal hemoglobin level from 10.2 to 28.6% in the peripheral blood over the same period of hydroxyurea therapy was also observed. Our results demonstrate that the increase in fetal hemoglobin levels in cells treated with hydroxyurea in vitro is comparable to the rise of fetal hemoglobin production following hydroxyurea therapy in these patients. On the contrary, these findings were not observed in three previously non-responsive sickle-cell patients. These results suggest that the changes in number of BFU-E colonies and fetal hemoglobin levels after in vitro exposure to hydroxyurea may be a useful approach to select sickle-cell patients who will respond to hydroxyurea therapy. Am. J. Hematol. 56:252–258, 1997.

INTRODUCTION

Sickling of red blood cells in patients with sickle-cell anemia (Hb SS) is caused by the formation of insoluble and rigid polymers of sickle hemoglobin under deoxygenated conditions. Fetal hemoglobin is known to interfere with polymerization of sickle hemoglobin and have beneficial effects in patients with sickle-cell anemia [1]. Several agents such as 5-azacytidine, hydroxyurea, and erythropoietin are known to stimulate fetal hemoglobin synthesis and have been used in sickle-cell patients to ameliorate severe pain episodes. Among these, hydroxyurea is currently the agent of choice because it is relatively safe and easy to administer. Previous open-labeled studies showed approximately 60% of patients treated with hydroxyurea responded with an elevated fetal hemoglobin level [2,3]. Recently, a double-blind multicenter trial showed a 44% reduction in the annual rate of

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pain episodes in patients with sickle-cell anemia treated with hydroxyurea [4]. There was also significant variation in the ability of patients with sickle-cell anemia to respond to hydroxyurea treatment. In addition, prolonged treatment is generally required to determine the clinical response and achieve maximal effect. Therefore, development of criteria to select patients who are most likely to respond to hydroxyurea treatment is desirable.

Various in vitro cell culture systems have been utilized to study the effects of fetal hemoglobin inducing agents on γ-globin gene expression. Utilizing a two-phase liquid culture system, Fibach et al. [5] demonstrated that sodium phenylacetate at pharmacologic concentrations can stimulate fetal hemoglobin production in erythroid progenitors from normal donors and patients with sickle-cell anemia or β-thalassemia. These results suggest that an in vitro cell culture assay might be a useful tool to predict the clinical response to phenylacetate and other fetal hemoglobin inducers.

Rapid regeneration of erythroid precursors following the cytoreduction phase of pharmacologic manipulation in vivo has been suggested as one of the mechanisms involved in the stimulation of fetal hemoglobin production. This mechanism of action has been invoked for cytotoxic agents such as 5-azacytidine and hydroxyurea [6–8], although direct evidence is lacking. We have previously demonstrated a strong inverse linear relationship between the total number of burst-forming unit-erythroid (BFU-E) colonies and fetal hemoglobin levels in sickle-cell patients treated with hydroxyurea [9]. These BFU-E study results suggest that hydroxyurea stimulates fetal hemoglobin synthesis possibly through alteration in cell cycle kinetics. This supports the hypothesis linking the induction of fetal hemoglobin to rapid erythroid regeneration. We extended our investigation in vitro utilizing mononuclear erythroid cells isolated from the peripheral blood of untreated sickle-cell patients. Erythroid progenitors were incubated in the presence of hydroxyurea in a methylcellulose culture system to assess the relationship between the different concentrations of this agent and the number of BFU-E colonies observed. Our data show that the total number of BFU-E colonies derived from the peripheral blood of patients with sickle-cell anemia decreased when the concentration of hydroxyurea increased in vitro.

In this study, we evaluated the relationship between BFU-E colony growth in response to hydroxyurea in vitro and the induction of fetal hemoglobin production both in culture and in sickle-cell patients treated with hydroxyurea.

MATERIALS AND METHODS

Subjects

Patients with sickle-cell anemia not previously treated with hydroxyurea with a history of at least six acute pain episodes during the 2 years prior to enrollment were invited to participate in the study. Patients with sickle-cell anemia who were non-responsive in previous hydroxyurea trial and had been off hydroxyurea for more than 4 months were also invited to participate in the in vitro studies. Informed consent was obtained from all patients following the University of South Alabama Institutional Review Board guidelines. Clinical response was defined by more than 50% decrease in the rate of pain episodes.

Blood Samples and Laboratory Studies

Ten milliliters (ml) of peripheral blood for BFU-E colony assay was drawn from each patient in a tube containing sodium heparin during the pre-study period to determine the optimal concentration of the effect of hydroxyurea on BFU-E colonies. At the time of enrollment in the study, peripheral blood samples were drawn from each patient for BFU-E colony assay prior to hydroxyurea treatment, then every 3–4 weeks while on treatment for a period of 5 months or until 4 weeks after the maximum tolerated dose of hydroxyurea was achieved. A complete blood count (CBC) with differential, reticulocyte count, fetal hemoglobin level, liver function (ALT, AST, LDH, total bilirubin), and renal function tests (creatinine and BUN) were determined on each patient prior to the initiation of hydroxyurea treatment.

Hydroxyurea Treatment and Follow-Up

The starting dose of hydroxyurea was 10 mg/kg/day. The daily dose was increased by 5 mg/kg every 3–4 weeks until either clinical or laboratory evidence of toxicity was observed or a maximum dose of 30 mg/kg/day was reached. After hydroxyurea treatment was started, each patient had a clinical evaluation every 2–4 weeks, a CBC with differential and reticulocyte count every 2 weeks, fetal hemoglobin level every 3–4 weeks, and liver and renal function tests every 8 weeks.

Measurement of BFU-E Colony

Mononuclear cells from the peripheral blood of each patient were isolated using Histopaque-1077 centrifugation (Sigma Biochemicals Co., St. Louis, MO). The cells were subsequently washed with Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum, 2 mM glutamine, and 10^{-4} M β-mercaptoethanol. The cells were mixed at a concentration of 3×10^5 cells/ml in 0.9% methylcellulose containing 30% fetal bovine serum, 2 mM glutamine, 1% deionized bovine serum albumin, 10^{-4} M β-mercaptoethanol, 10 ng of human recombinant interleukin-3, and 3 U/ml of human recombinant erythropoietin. The cells were incubated in multiwell tissue culture plates in a humidified atmosphere containing 5% CO_2, at 37°C. Different pharmacologically achievable concentrations of hydroxyurea (25, 50,

TABLE I. Effects of Different Concentrations of Hydroxyurea on BFU-E Colony Growth and Fetal Hemoglobin Production in Previously Untreated Sickle-Cell Patients†

	Concentrations at HU (μM)									
	0		25		50		75		100	
Patient no.	BFU-E	Hb F	BFU-E	Hb F	BFU-E	Hb F	BFU-E	Hb F	BFU-E	Hb F
1	126	4	40	5	24	6	10	11	2	20
2	194	2	157	3	82	6	39	8	27	12
3	127	6	89	13	78	19	40	23	0	25
4	150	12	121	17	69	19	23	21	1	25
5	171	1	85	3	38	7	21	10	6	15
Mean ± SEM	153.7 ± 13.0	5.1 ± 1.9	98.3 ± 19.6	8.2 ± 2.9	58.2 ± 11.5	11.4 ± 3.1	26.7 ± 5.7	14.7 ± 3.1	7.2 ± 5.1	19.4 ± 2.6
P value*	—	—	0.0003	0.0347	0.0001	0.0003	0.0001	0.0001	0.0001	0.0001

†HU = hydroxyurea. Shown is the number of BFU-E Colonies per 3×10^5 mononuclear cells. Hb F = fetal hemoglobin in percent (%).
**P* value represents a comparison to 0 μM of hydroxyurea concentration in the number of BFU-E colonies and in the fetal hemoglobin level.

75, and 100 μM) were added to quadruplicate wells in the culture plates on day 0 of the study period. BFU-E colonies were counted on day 14 by an inverted microscope and harvested for fetal hemoglobin determination or RNA analysis.

Fetal Hemoglobin Determination

Peripheral blood fetal hemoglobin levels were measured before and after hydroxyurea treatment by standard hemoglobin electrophoresis using cellulose acetate membranes at alkaline pH. The fetal hemoglobin level in the BFU-E colonies harvested on day 14 was measured by an alkaline denaturation method described previously [9]. In brief, the supernatant obtained after treating the cells with 1.2 N NaOH and a saturated ammonium sulfate solution was quantitated spectrophotometrically.

Haplotype Determination

Genomic DNA was isolated from peripheral blood leukocytes by standard methods. The haplotype was determined by polymerase chain reaction amplification techniques, using previously designed primer pairs [10]. Seven amplified fragments containing polymorphic sites along the β-globin gene cluster were digested with restriction endonucleases and electrophoresed on a 2% agarose gel. The banding patterns previously described [11], consistent with the four major haplotypes identified in the sickle-cell patient population, were identified on ethidium bromide stained gels.

RNA Analysis

Total cytoplasmic RNA was isolated by the method of Chomczynski and Sacchi [12]. RNA concentrations and purity were assessed by spectrophotometry. Globin mRNA was analyzed by RNase protection with the following probes: $pT_{7\gamma}$, linearized with *BSTEII* to give a 170-bp protected fragment and a human actin probe (Ambion, Austin, TX) that yields a 245-bp protected fragment. RNA (2 μg) was hybridized overnight at 45°C with 10^6 cpm of each radiolabeled probe. After digestion with RNase A, the protected fragments were separated on a 6% polyacrylamide-8M urea gel and autoradiographed without an intensifying screen. Human Aγ and actin mRNA were quantitated using a Bio-rad GS-250 Phosphor Imager (Bio-Rad, Richmond, CA). Human γ-globin gene mRNA production was calculated as a ratio of γ/actin mRNA.

Statistical Analysis

The statistical methods used in the analysis include paired *t*-test, two sample *t*-tests, one-way and two-way analysis-of-variance, correlation analysis, fitting of general linear statistical models, and testing for parallelism, equal intercepts, and coincidence. The computations were performed using SAS statistical software (Statistical Analysis System, Cary, NC).

RESULTS

Effects of Different Concentrations of Hydroxyurea on BFU-E Colony Formation and Fetal Hemoglobin Production In Vitro

In the in vitro period, peripheral blood samples were drawn to determine the range of the cytotoxic effect of hydroxyurea. Five patients with sickle-cell anemia not previously treated with hydroxyurea were enrolled in the study (patients 1 to 5). Pharmacologically achievable concentrations of hydroxyurea ranging from 25 to 100 μM were analyzed in culture. The relationship between the mean number of BFU-E colonies and the mean fetal hemoglobin levels in erythroid progenitors exposed to different concentrations of hydroxyurea in vitro is shown in Table I. For each of the patients studied, as the concentration of hydroxyurea increased from 0 to 100 μM, the number of BFU-E colonies decreased from an average of 153.7 per 3×10^5 mononuclear cells without hydroxyurea, to 7.2 per 3×10^5 mononuclear cells at 100μM hydroxyurea concentration (Table I). Similarly, the fetal hemoglobin level increased from an average of 5.1 to 19.4% (an over 3-fold increase) at the 100 μM con-

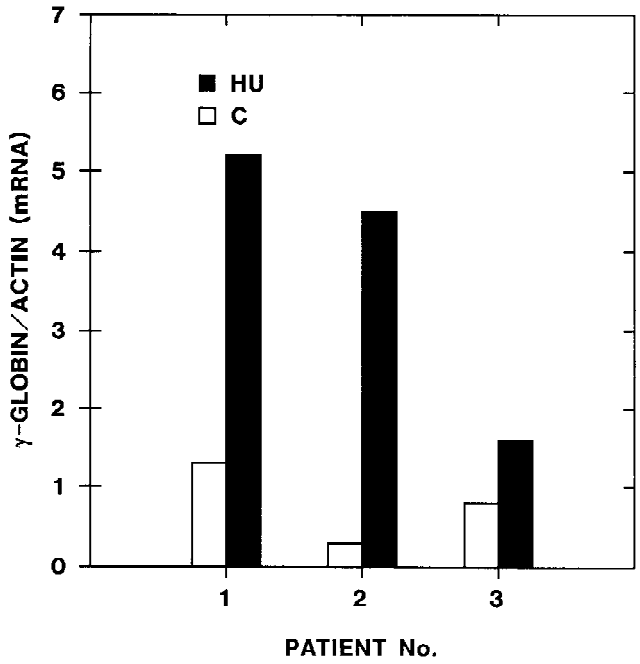

Fig. 1. Induction of γ gene mRNA production by hydroxyurea in culture. Total cytoplasmic RNA was analyzed by RNase protection assay to quantitate γ mRNA levels in BFU-E colonies without (C) and in the presence of hydroxyurea (HU). γ-mRNA production before and after treatment is shown in the open and closed bars, respectively. Three of the four patients had an increased γ-gene expression in response to hydroxyurea exposure.

centration of hydroxyurea in the five subjects tested (Table I). The correlation coefficient between the number of BFU-E colonies and different levels of hydroxyurea is −0.89 ($P < 0.0001$). The correlation coefficient between the fetal hemoglobin level and different concentrations of hydroxyurea is +0.67 ($P = 0.0003$). A significant inverse relationship ($r = -0.57$, $P = 0.0027$) was observed between the number of BFU-E colonies and the fetal hemoglobin level. The change in the number of BFU-E colonies is significantly different between 0 μM and other concentrations (25, 50, 75, and 100 μM) of hydroxyurea. The same is true for the fetal hemoglobin level except at the concentration of 25 μM (see Table I).

Changes in γ-globin gene expression in the BFU-E colonies were correlated at the transcriptional level by quantitation of mRNA production in response to culture in 100 μM hydroxyurea by RNase protection assay. The results for γ-globin mRNA production in 3 previously untreated patients are shown in Figure 1. All 3 patients tested showed changes in γ-mRNA as a ratio to actin, from an average of 0.800 ± 0.29 to an average of 3.767 ± 1.10. The increase of γ-mRNA production ranged from 2- to 15-fold. Patient 3 showed the least change (2-fold) in γ-gene expression with hydroxyurea treatment.

TABLE II. Summary of Clinical and Laboratory Data of All Patients*

Patient no.	Age (years)	Sex	Hb (g/dl) Pre	Hb (g/dl) Post	Hb F (%) Pre	Hb F (%) Post	Clinical response
1	19	F	9.5	11.4	15	33	Yes
2	16	M	8.7	11.3	7	42	Yes
3	17	F	8.5	9.8	8	25	Yes
4	17	M	9.8	11.4	21	32	Yes
5	16	M	8.8	11.0	0	17	Yes
6	25	M	9.9	10.5	10	9	No
7	31	M	10.0	9.3	<2	<2	No
8	29	M	9.7	7.8	3	3	No

*Pre = hemoglobin concentration and fetal hemoglobin level before hydroxyurea treatment; Post = hemoglobin concentration and fetal hemoglobin level at the end of the study period after hydroxyurea therapy.

Correlation in In Vitro BFU-E Colony Formation With Fetal Hemoglobin Induction in Patients Treated With Hydroxyurea

Hydroxyurea therapy was initiated after two in vitro erythroid progenitor assays were completed for each of the 5 patients enrolled in the study (patients 1 to 5). A summary of clinical and laboratory data before and after hydroxyurea therapy is shown in Table II. There were 3 males and 2 females, from 16 to 19 years of age. All patients were in the steady state and received no blood transfusions for at least 4 months before the samples were collected. Haplotype studies show that patients 1 and 2 are Bantu, patients 3 and 4 are Benin, and patient 5 is CAR. All 5 patients had a significant reduction in frequency of pain episodes. The frequency of acute painful episodes decreased from 6.1 ± 1.2 to 3.0 ± 1.4 in a 12-month period before and after hydroxyurea therapy, $P = 0.0027$. Changes in the number of BFU-E colonies and fetal hemoglobin levels before and after the start of hydroxyurea treatment in the 5 patients studied are presented in Figure 2. A similar scenario was observed with mononuclear cells obtained from patients at various time points during hydroxyurea treatment. The BFU-E colonies generated decreased from 153.7 (at week 0) to 2.0 per 3×10^5 mononuclear cells, at greater than 9 weeks of hydroxyurea treatment (Fig. 2). The relationship between the mean number of BFU-E colonies in culture and the peripheral blood fetal hemoglobin levels achieved in these 5 patients, before and after hydroxyurea treatment, is shown in Figure 2. The data show that the number of BFU-E colonies is gradually decreased while the fetal hemoglobin level is gradually increased over a greater than 9-week period during hydroxyurea treatment. Our results demonstrate that the increase of fetal hemoglobin levels in erythroid precursor cells (from 5.1 to 19.4%) grown in the presence of hydroxyurea in vitro is significant ($P = 0.0022$), and is similar to the increase of fetal hemoglobin production (from 10.2 to 28.6%) in the pe-

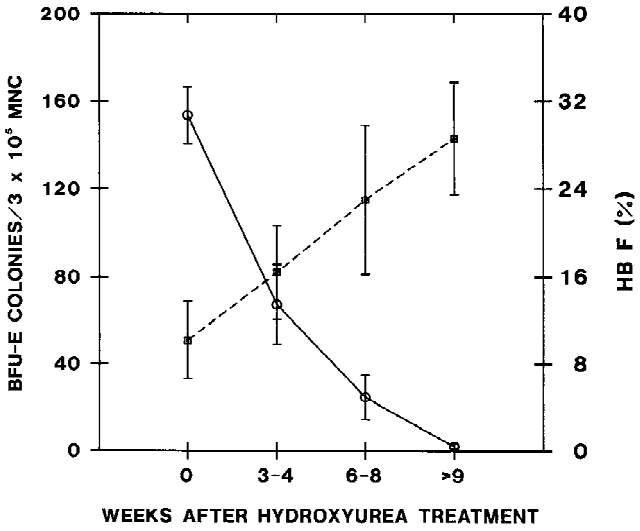

Fig. 2. Correlation between the number of BFU-E colonies in culture and the peripheral blood fetal hemoglobin levels achieved before and after hydroxyurea treatment in 5 patients. Peripheral mononuclear cells of each were cultured as described in Materials and Methods. The results were expressed as the number of BFU-E colonies per 3 × 10^5 mononuclear cells in culture plates (○—○) and fetal hemoglobin levels as percent of total hemoglobin in the peripheral blood (■- - -■).

ripheral blood following hydroxyurea treatment in these 5 patients ($P = 0.0196$).

Lack of Cytotoxic Effect of Hydroxyurea on BFU-E Colony Growth in Non-Responsive Patients

Three patients with sickle-cell anemia who previously failed to respond to hydroxyurea were recruited for the in vitro cell culture experiment to assess the effects of different concentrations of hydroxyurea on BFU-E colony formation (patients 6, 7, and 8). Patients 6 and 7 had been off hydroxyurea for 1 year, and patient 8 had been off hydroxyurea for 4 months before the blood samples for BFU-E colony assay were obtained. The clinical and laboratory data for these patients are shown in Table II. Total hemoglobin concentration, reticulocyte count, and WBC were 9.9 ± 0.1 g/dl, 13.9 ± 1.0%, and 10.7 ± 1.4 × 10^9/1, respectively, before hydroxyurea treatment, and 8.9 ± 0.3 g/dl, 13.1 ± 2.5%, and 13.4 ± 2.4 × 10^9/1, respectively, at the time of BFU-E colony analyses in vitro. There are no significant differences for the CBC and reticulocyte count between the baseline (pre-hydroxyurea treatment) values and at the time of BFU-E culture for these 3 patients. The effects of different concentrations of hydroxyurea on BFU-E colony growth and fetal hemoglobin production are shown in Table III. The number of BFU-E colonies decreased minimally after treatment with hydroxyurea between the concentrations 0 and 50 μM and decreased only modestly at concentrations of 75 and 100 μM (from 209.7 to 112.1 per 3 × 10^5 mononuclear cells). Similarly, there was a lack of fetal hemoglobin induction (from 3.4 to 4.6%). These results demonstrate significant differences in the changes of the number of BFU-E colonies at varying concentrations of hydroxyurea for the 5 patients who had a favorable response to hydroxyurea therapy vs. the 3 patients who did not respond to therapy as depicted in Figure 3 ($P < 0.0001$). Similarly, a significant difference in the ability to induce fetal hemoglobin product at different hydroxyurea concentration in vitro was observed for the two groups ($P < 0.0001$).

DISCUSSION

The benefit of hydroxyurea treatment in patients with sickle-cell anemia is thought to be primarily due to increased fetal hemoglobin production. However, other mechanisms may also play a role in the effects of this compound. Administration of hydroxyurea to patients with sickle-cell anemia increases fetal hemoglobin, F reticulocyte count, fetal hemoglobin containing red blood cells, mean corpuscular volume, mean corpuscular hemoglobin concentration, total hemoglobin concentration, erythrocyte survival, and oxygen affinity. The increase in the peripheral blood total hemoglobin concentration despite the marked decrease in the number of circulating BFU-E colonies in 5 patients who responded to hydroxyurea is probably due to increased survival of red cells containing fetal hemoglobin (F cell) [13,14]. In addition, hydroxyurea treatment may induce the following changes on red blood cells: increased total cation content, with improvement of deformability and hydration status [13,14] and decreased adherence to vascular endothelial cells [15]. Less is known about the molecular mechanism involved in γ-gene induction by hydroxyurea. Previous studies by Platt et al. [16] showed that demethylation of the γ-globin genes accompanies increased γ-globin gene activity in patients with sickle-cell disease treated with hydroxyurea. Recent data from Huang et al. [17] suggest that hydroxyurea may induce both γ-globin and α-globin gene expression at the transcriptional level, demonstrated by increased mRNA production in sickle-cell patients treated with hydroxyurea. Thus, the experimental data supports a multi-level mechanism of action for fetal hemoglobin induction and the anti-sickling effects of hydroxyurea therapy.

A widely proposed mechanism of the action of hydroxyurea is its ability to interrupt DNA synthesis in rapidly dividing late erythroid precursors by inhibition of the enzyme ribonucleotide reductase, which leads to transient arrest of hematopoiesis [18]. Recovery from such an arrest is associated with stress hematopoiesis, consisting of recruitment of earlier progenitors that have re-

TABLE III. Effects of Different Concentrations of Hydroxyurea on BFU-E Colony Growth and Fetal Hemoglobin Production in Non-Responsive Patients*

	Concentrations of HU (μM)									
	0		25		50		75		100	
Patient no.	BFU-E	Hb F	BFU-E	Hb F	BFU-E	Hb F	BFU-E	Hb F	BFU-E	Hb F
6	232.3	2	210	2.7	194.3	4	173.3	5	110	5
7	192.7	4	196	3	177.7	4	171.6	4	113.3	4
8	204.0	4	206	4	192	5	188.9	5	113	5
Mean ± SEM	209.7 ± 11.8	3.4 ± 0.7	204.0 ± 4.2	3.3 ± 4.2	188.0 ± 5.2	4.4 ± 0.4	177.8 ± 5.5	4.6 ± 0.4	112.1 ± 1.1	4.6 ± 0.4

*HU = hydroxyurea; Hb F = fetal hemoglobin in percent (%). Shown is the number of BFU-E colonies per 3×10^5 mononuclear cells.

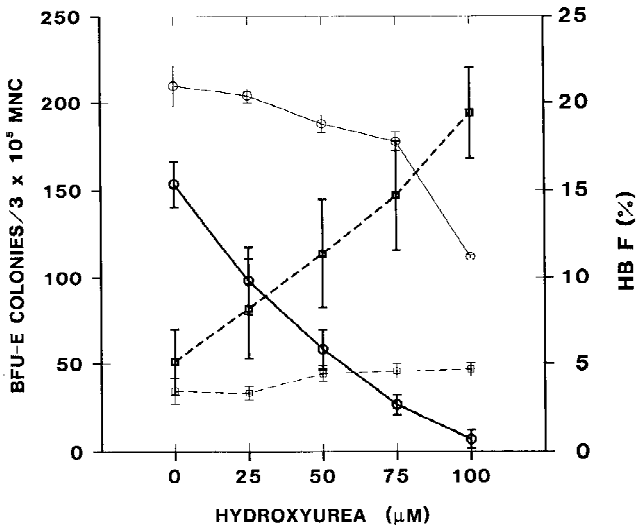

Fig. 3. Comparison of BFU-E colonies and fetal hemoglobin levels in the presence of varying concentrations of hydroxyurea in the cell culture system between responding and non-responding sickle-cell patients. Mononuclear cells were cultured as described in Materials and Methods. The results were expressed as the number of BFU-E colonies per 3×10^5 mononuclear cells (○—○ for responders and ○—○ for non-responders) and fetal hemoglobin levels (Hb F) as percent of total hemoglobin (□- - -□ for responders and □- - -□ for non-responders). Data are the mean ± SEM of three independent experiments. Significant differences in the changes of the number of BFU-colonies and fetal hemoglobin level at different hydroxyurea concentrations are observed between the responders and non-responders.

tained their fetal hemoglobin producing capacity. Thus, mature erythrocytes are derived from progenitor cells that continue to actively express a γ-globin gene program, resulting in increased fetal hemoglobin production [19]. The entire mechanism whereby erythroid regeneration stimulates fetal hemoglobin production remains to be determined.

Previous and more recent clinical data demonstrated a variable response in patients with sickle-cell anemia to hydroxyurea therapy. Fetal hemoglobin levels can be increased in the majority of sickle-cell patients treated with hydroxyurea over time [2]. However, the magnitude of the increase in fetal hemoglobin production varies, ranging from approximately 2% to more than 20% [2,3]. The relative increase in fetal hemoglobin production has been shown to be more dependent on the initial level of fetal hemoglobin, reticulocyte count, plasma hydroxyurea levels, and initial white blood cell count, rather than on β-globin gene haplotype or α-globin gene number [2]. In this study, there are no significant differences on the initial fetal hemoglobin level, total hemoglobin concentration, reticulocyte count, and white blood cell count between the responders and non-responders. Hence, none of these factors has predictive power for response to hydroxyurea. A more detailed analysis of these parameters in a larger sample size may clarify which factors are most useful for predicting a clinical response to therapy.

Based on the experimental data available on the cytotoxic effects of hydroxyurea on CFU-E colonies, a relatively simple in vitro culture system was utilized to analyze the effects of hydroxyurea on BFU-E colony formation. Our initial studies established a dose response curve for hydroxyurea vs. BFU-E colony growth. We observed a consistent decrease in BFU-E colony number as the concentration of hydroxyurea increased to 100 μM. This concentration of hydroxyurea used in culture is achievable pharmacologically in patients treated with this agent. These findings support a substantial role for cytotoxicity in the mechanism of action for fetal hemoglobin induction by hydroxyurea. The findings in this study suggest in vitro BFU-E colony assay is a valuable tool for predicting responsiveness to hydroxyurea treatment. Alternatively, this may be a method to determine the dose-related toxicity for patients unresponsive to recommended dose of hydroxyurea (10–30 mg/kg/day).

Hydroxyurea was discontinued for more than 4 months (mean = 8 months) before the blood samples were obtained for BFU-E colony assay in 3 non-responsive patients. The CBC and reticulocyte counts were similar between the pre-hydroxyurea treatment (baseline) value and at the time of BFU-E colony assay analysis. These findings suggest that the hematopoietic activity was not affected by previous hydroxyurea treatment, and the marrow function most likely had returned to pre-treatment conditions when the cell culture study was performed. We observed a higher number of BFU-E

colonies in the non-responders, which may be due to the fact that these blood samples were shipped overnight from another institution before being processed. All blood samples from the 5 responders were collected locally and were processed immediately. It has been observed that the number of BFU-E colonies is generally greater in blood samples processed overnight compared to those processed immediately in our cell culture system (unpublished observation). However, the difference is not statistically significant.

Data obtained in the present study show that, in our in vitro cell culture system as the concentration of hydroxyurea is increased, the total number of BFU-E colonies derived from the peripheral blood of sickle-cell patients is decreased. The data also reveal that the decrease of the total number of BFU-E colonies correlates with increased fetal hemoglobin levels. Our results demonstrate a strong inverse relationship between the number of BFU-E colonies and the fetal hemoglobin level when cells were grown in pharmacologically achievable concentrations of hydroxyurea. These data also suggest that the inhibitory effect of hydroxyurea on BFU-E colony growth and subsequent induction of fetal hemoglobin is significantly greater in patients who responded to hydroxyurea therapy than in non-responsive patients. A possible explanation of this phenomenon is that effective suppression of BFU-E colony growth enhances premature commitment of earlier progenitors and leads to accelerated erythropoiesis; as a result, F-cell production is induced. Conversely, lack of an inhibitory effect of hydroxyurea on BFU-E colony growth results in stimulation for rapid erythroid regeneration and minimal increases in fetal hemoglobin production.

Our observations suggest that the changes in the number of BFU-E colonies and fetal hemoglobin level after in vitro hydroxyurea exposure in this cell culture system may be useful for selecting patients who will respond to hydroxyurea treatment. Further studies with a larger patient population are necessary to conclude whether this system can be used as a reliable predictor of a clinical response to hydroxyurea therapy in patients with sickle-cell anemia.

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REFERENCES

1. Charache S: Fetal hemoglobin, sickling and sickle cell disease. Adv Pediatr 37:1, 1990.
2. Charache S, Dover GH, Moore RD, Eckert S, Ballas SK, Kashy M, Milner PFA, Orringer EP, Phillips G, Platt OS, Thomas GH: Hydroxyurea effects on hemoglobin F production in patients with sickle cell anemia. Blood 79:2555, 1992.
3. Rodgers GP, Dover GJ, Noguchi CT, Schechter AN, Nienhuis AW: Hematologic responses of patients with sickle cell disease to treatment with hydroxyurea. New Engl J Med 322:1037, 1990.
4. Charache S, Terrin M, Moore R, Dover G, Barton F, Eckert S, McMahon R, Bonds D: Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. New Engl J Med 332:1317, 1995.
5. Fibach E, Prasanna P, Rodgers GP, Samid D: Enhanced fetal hemoglobin production by phenylacetate and 4-phenylbutyrate in erythroid precursors derived from normal donors and patients with sickle cell anemia and β-thalassemia. Blood 82:2203, 1993.
6. DeSimone J, Heller P, Hall L, Zwiers D: 5-azacytidine stimulates fetal hemoglobin synthesis in anemic baboons. Proc Natl Acad Sci. USA 79:4428, 1988.
7. Lavelle D, DeSimone J, Heller P, Zwiers D, Hall L: On the mechanism of fetal hemoglobin elevations in the baboon by erythropoietic stress and pharmacologic manipulation. Blood 67:1083, 1986.
8. Charache S: Hydroxyurea as treatment for sickle cell anemia. Hematol Clin North Am 5:571, 1991.
9. Mankad VN, Baliga BS, Phillips K, Shah AK, Yang YM: Relationship of burst-forming-unit-erythroid progenitors and their DNA-synthesis stage to fetal hemoglobin levels in hydroxyurea-treated patients with sickle cell anemia. Am J Hematol 46:259, 1994.
10. Sutton M, Bouhassira EE, Nagel RL: Polymerase chain reaction amplification applied to the determination of β-like globin gene cluster haplotypes. Am J Hematol 32:66, 1989.
11. Nagel RL, Labie D: DNA haplotype and the β globin gene. Sixth Conference on Hemoglobin Switching, Arlie House, Virginia, September 24–27, 1988.
12. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987.
13. Ballas SK, Dover G, Charache S: Effect of hydroxyurea on the rheological properties of sickle erythrocytes in vivo. Am J Hematol 32:104, 1989.
14. Goldberg M, Brugnara C, Dover G, Schapira L, Charache S, Bunn HF: Treatment of sickle cell anemia with hydroxyurea and erythropoietin. New Engl J Med 323:366, 1990.
15. Adragna N, Fonseca P, Lauf P: Hydroxyurea affects cell morphology, cation transport, and red blood cell adhesion in cultured vascular endothelial cells. Blood 83:553–560, 1994.
16. Platt O, Orkin S, Dover G, Beardsley GP, Miller B, and Nathan D: Hydroxyurea enhances fetal hemoglobin production in sickle cell anemia. J Clin Invest 74:652, 1984.
17. Huang SZ, Fibach E, Gu XF, Schechter AN, Rogers GP, Zend YT: Hydroxyurea enhances β-globin expression and decreases aberrant splicing in cultured β-thalassemia cells. Blood 86:482a, 1995.
18. Dover GJ, Humphries RK, Moore JG, Ley TJ, Young NS, Charache S, Nienhuis AW: Hydroxyurea induction of hemoglobin F production in sickle cell disease: Relationship between cytotoxicity and F cell production. Blood 67:735, 1986.
19. Papayannopoulou TH, Vichinsky E, Stamatoyannopoulos G: Fetal Hb production during acute erythroid expansion, I. Observation in patients with transient erythroblastopenia and postphlebotomy. Br J Haematol 44:535, 1980.